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Low-field nuclear magnetic resonance relaxation study of thermal effects on milk proteins

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Summary. A recently described nuclear magnetic resonance (NMR) method was evaluated for its usefulness in studying thermal effects on milk proteins. The increase in water proton T_2 relaxation rate observed during thermal treatment of aqueous whey protein solutions above the denaturing onset temperature paralleled results obtained with the standard Rowland (1938) method. The influence of milk constituents on NMR characteristics was analysed. The NMR response increased with the ionic strength and the addition of caseinate or casein micelles. The relevance of the T_2 relaxation probe for studying thermal modifications of milk proteins is discussed. It is proposed to apply the NMR method for determining either reversible or irreversible thermal denaturation of whey proteins in model systems.

The functional properties of a food product, governed by the state of the proteins, are a key factor in food technology. Low resolution nuclear magnetic resonance (NMR) spectroscopy can be an alternative to current methods for studying protein thermal denaturation (Pumpernik *et al.* 1975; Oakes, 1976*a*, *b*; Blicharska & Rydzy, 1979; Rydzy & Skrzynski, 1980; Goldsmith & Toledo, 1985). In order to eliminate the effect of temperature on relaxation, we proposed for the study of protein thermal denaturation by NMR spectroscopy a method in which the measurements are always effected at the same temperature (Lambelet *et al.* 1989). Under these conditions thermal denaturation of diamagnetic whey proteins was expressed by an increase in water proton transverse relaxation rate ($1/T_2$) (Lambelet *et al.* 1989) while a decrease in this value was recorded during thermal denaturation of paramagnetic proteins such as Fe-saturated transferrins (Lambelet *et al.* 1991).

Thermal denaturation of whey proteins is also governed by 'environmental' factors (Mulvihill & Donovan, 1987), such as ionic strength (Mulvihill & Kinsella, 1988), specific ions such as Ca^{2+} (De Wit, 1981; Mulvihill & Kinsella, 1988) and casein (Reddy & Kinsella, 1990). The present paper describes the influence of these factors on the NMR response recorded during the denaturation of whey proteins, and discusses the potential and limitations of the NMR method for investigating thermal effects on proteins in casein, whey protein isolates and model milk samples.

MATERIALS AND METHODS

Materials

Whey protein isolate (WPI) prepared by hydrophilic ion-exchange chromatography was purchased from Le Sueur Isolates (Le Sueur, MN 96058, USA). The

product had a protein content of 90·4%. Freeze-dried κ-casein was purchased from Sigma (CH-9470 Buchs).

Dialysed skim milk

Raw cows' milk was defatted by centrifugation at 4800 ***g*** and 30 °C for 30 min and dialysed at 4 °C using 6000–8000 Da membranes against 12 vol. distilled water for ~ 2·5 h. This last operation was repeated six times. The freeze-dried powder had a protein content of 69·2% of which 59·3% was casein (Kjeldahl N × 6·38 of the fraction precipitated at pH 4·6 with an acetate buffer).

Sodium caseinate

Sodium caseinate was prepared from raw skim milk by precipitation at pH 4·6 with M-HCl followed by centrifugation at 4800 ***g***, dissolution of the deposit in water at pH 7·5 with M-NaOH, reprecipitation at pH 4·6, centrifugation at 4800 ***g***, washing of the deposit with demineralized water and resolubilization at pH 7·5 with M-NaOH. The solution was spray dried and the powder obtained had a protein content of 81·6%.

Casein micelles

Casein micelles were isolated from raw skim milk by ultracentrifugation at 100 000 ***g*** and 10–30 °C for 90 min. The micelles obtained were collected and freeze dried. They had a protein content of 85·5%.

Sample preparation

Protein solutions were prepared by mixing at room temperature the proteins (whey proteins alone or in admixture with casein micelles or sodium caseinate) with either distilled water or with aqueous solution of KCl or $CaCl_2$. After their pH had been adjusted to 7 with 0·1 M-NaOH, the solutions were degassed under vacuum. Denaturation was achieved (1) by heating the sample for 30 min successively at increasing temperatures between 20 °C and 110 °C (10 °C interval) or (2) by heating the sample at a constant temperature > 50 °C directly in the NMR cavity. In the first case the NMR measurements were carried out following each thermal treatment, on samples kept at 20 °C for 30 min. In the second case NMR results were continuously recorded with time.

Determination of protein content and protein denaturation (Rowland, 1938)

Total N was determined according to the method of Association of Official Analytical Chemists (1980). The protein content was calculated by subtracting the non-protein N (NPN; measured after precipitation in the presence of 12% trichloroacetic acid) from the total N. Whey protein N (WPN) was calculated by subtracting NPN from non-casein N (measured after precipitation at pH 4·6). The percent denaturation was calculated by comparing the WPN in heat-treated and in raw samples.

$$\text{Denaturation, \%} = \frac{\text{WPN}_{\text{raw}} - \text{WPN}_{\text{treated}}}{\text{WPN}_{\text{raw}}} \times 100$$

NMR measurements

NMR measurements were run on a Minispec PC20 (Bruker Physik AG, 7501 Karlsruhe, Germany) at a resonance frequency of 20 MHz. The spectrometer was coupled to an HP 950 computer allowing automatic data acquisition. T_2 relaxation

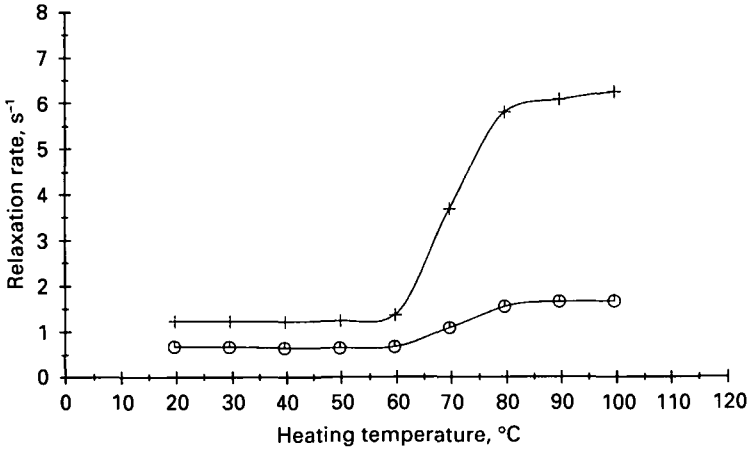

Fig. 1. Water proton T_2 relaxation rates in whey protein isolate (WPI) solutions heated for 30 min successively at increasing temperatures between 20 and 100 °C (10 °C interval): ○, 3% WPI solution; +, 10% WPI solution.

times were determined from the spin echoes decay curve (169 echoes) observed with a Carr–Pursell–Meiboom–Gill pulse sequence. T_2 relaxation times were calculated by performing nonlinear regression of exponential to the decay curves.

RESULTS

As the T_2 relaxation probe has been shown to be more sensitive than T_1 for measuring denaturation of diamagnetic proteins by heat (Oakes, 1976*a*, *b*; Lambelet *et al.* 1989), T_2 was exclusively used in the present study. Whatever the thermal treatment applied, water proton T_2 relaxation in all milk models investigated could be analysed in terms of a single exponential.

Whey proteins aqueous solutions

When an aqueous solution of WPI (3 or 10% w/v) was heated for 30 min at temperatures ranging from 20 to 100 °C and then cooled to 20 °C, a rise in water proton T_2 relaxation rate was recorded after thermal treatment between 60 and 80 °C (Fig. 1); the variation in T_2 relaxation rate increased with the protein concentration. In contrast, no modification of this rate was observed outside this temperature range.

Thermal denaturation measured by the standard Rowland (1938) method while heating a 10% (w/v) WPI aqueous solution at 60, 67, 72 or 75 °C is displayed in Fig. 2. Whatever the temperature of the thermal treatment between 67 and 75 °C, ~ 98% denaturation was observed after 10 h heating.

The denaturation rate increased with temperature in the range 60–75 °C. The T_2 relaxation rates jointly measured during heating these whey protein solutions showed the same tendency (Fig. 3). But for small differences due to the dependence of relaxation rate on the temperature, the relaxation rates recorded after 10 h were identical for any heating temperature between 67 and 75 °C. Moreover, even at 60 °C the NMR signal reached the same level as those measured at higher temperatures, but this was after ~ 25 h heating (results not shown). The variation of the NMR response with time was slow at 60 °C, intermediate at 67 °C and rapid at 72 or 75 °C.

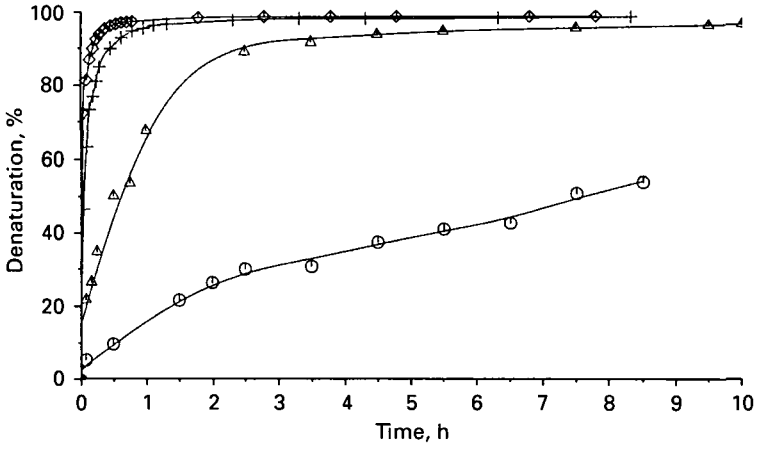

Fig. 2. Time course of denaturation as determined by the method of Rowland (1938) in 10% (w/v) whey protein isolate solutions heated at ○, 60 °C; △, 67 °C; +, 72 °C; ◇, 75 °C.

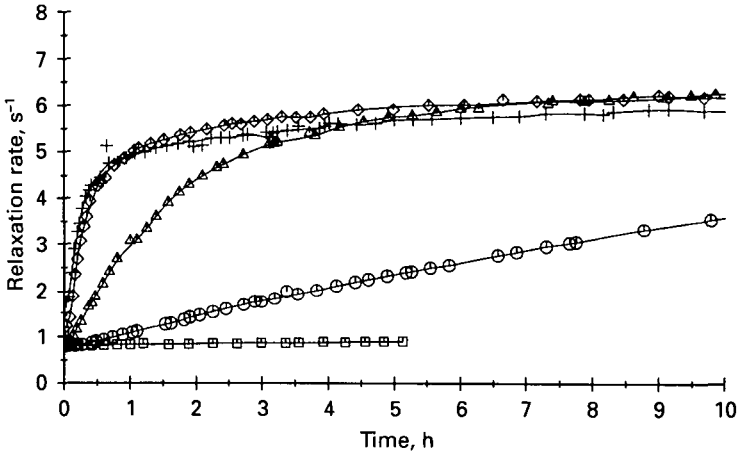

Fig. 3. Time course of water proton T_2 relaxation rates in 10% (w/v) whey protein isolate solutions heated at □, 50 °C; ○, 60 °C; △, 67 °C; +, 72 °C; ◇, 75 °C.

At 50 °C, i.e. below the onset denaturing temperature, no change in the NMR signal was observed.

Whey proteins in KCl or $CaCl_2$ solutions

Successive heatings at temperatures between 20 and 100 °C of 10% (w/v) WPI in 10 mM-KCl, 100 mM-KCl or 10 mM-$CaCl_2$ solutions gave NMR responses (Fig. 4) similar to those recorded in pure aqueous solutions (Fig. 1). An increase in water proton T_2 relaxation rate was thus recorded after thermal treatment between 60 and 80 °C. The NMR response, however, was slightly enhanced in the presence of the salts.

The effects of these salts on isothermal denaturation (67 °C) determined by the Rowland (1938) method are summarized in Fig. 5. The same denaturation level (~ 98%) was found after 10 h heating whether or not the solution contained a salt. Whilst a tiny decrease of the initial denaturation rate was observed with 100 mM-KCl, a small increase in this rate was recorded in the presence of 10 mM-KCl or 33 mM-$CaCl_2$.

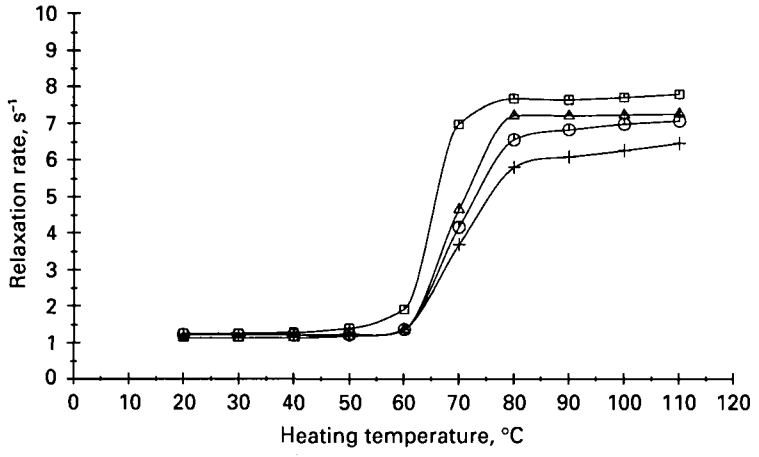

Fig. 4. Water proton T_2 relaxation rates in 10% (w/v) whey protein isolate solutions of different ionic strengths heated for 30 min successively at increasing temperatures between 20 and 110 °C (10 °C interval): +, aqueous solution; ○, 10 mM-KCl; △, 100 mM-KCl; □, 33 mM-$CaCl_2$.

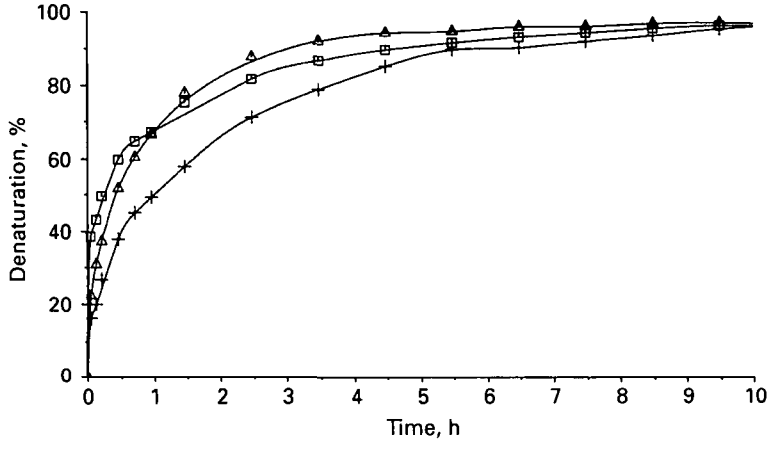

Fig. 5. Time course denaturation (Rowland, 1938) in 10% (w/v) whey protein isolate solutions of different ionic strengths heated at 67 °C: △, 10 mM-KCl; +, 100 mM-KCl; □, 33-mM $CaCl_2$.

These salts, however, more drastically modified the NMR response recorded during isothermic heating (Fig. 6). The initial increase in the NMR signal and the level of this signal measured after 10 h heating at 67 °C were higher in 10 mM-KCl than in pure aqueous solution. These two values were still higher when the solution contained 100 mM-KCl. In the presence of 33 mM-$CaCl_2$ the initial slope of the NMR signal was the largest, although the final level reached by this signal after 10 h heating was similar to that measured in 100 mM-KCl solutions (Fig. 6). It is worth noting on the other hand that heating WPI in 10 mM-KCl at 67 °C did not induce gelation while a gel was formed in 100 mM-KCl or 33 mM-$CaCl_2$.

Mixtures of whey proteins and casein in water solutions

T_2 water proton relaxation rate in 12% (w/v) sodium caseinate water solution remained constant at 2·2 s^{-1} during heating for 30 min at 90 °C. The T_2 relaxation rates in 3% (w/v) WPI solutions containing various amounts of sodium caseinate

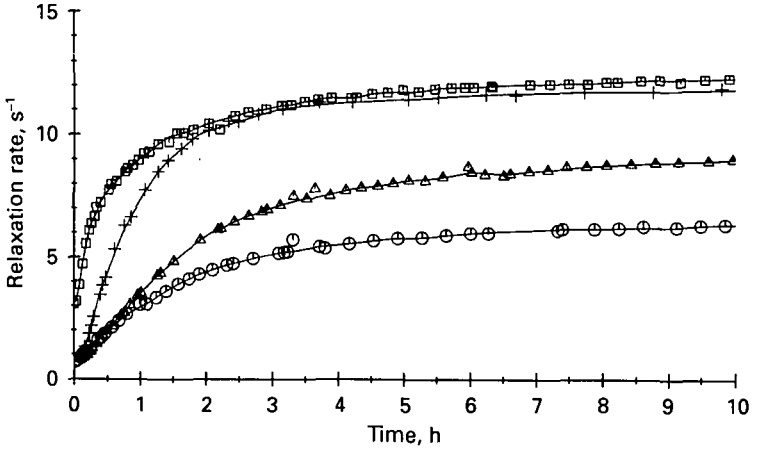

Fig. 6. Time course of water proton T_2 relaxation rates in 10% (w/v) whey protein isolate solutions of different ionic strengths heated at 67 °C: ○, aqueous solution; △, 10 mM-KCl; +, 100 mM-KCl; □, 33 mM-$CaCl_2$.

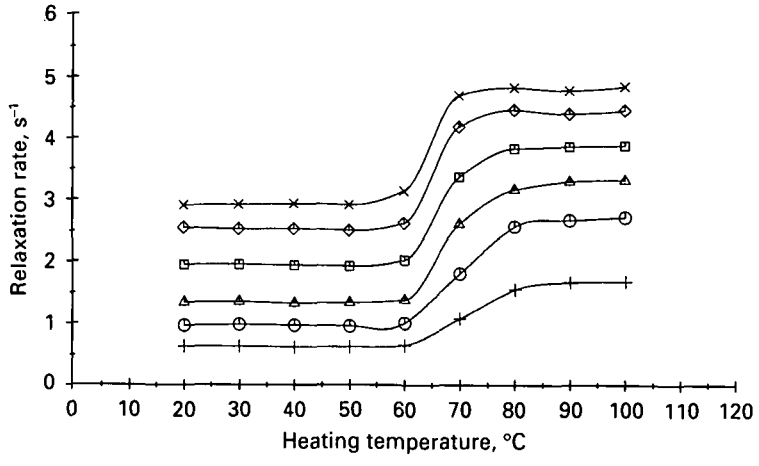

Fig. 7. Water proton T_2 relaxation rates in 10% (w/v) whey protein isolate solutions with added sodium caseinate heated for 30 min successively at increasing temperatures between 20 and 100 °C (10 °C interval). Addition of caseinate (w/v): +, no addition; ○, 3%; △, 5%; □, 8%; ◇, 10%, ×, 12%.

(0–12% w/v) increased linearly with the content of caseinate. For all these solutions, however, an increase in T_2 water proton relaxation rate was observed following heating in the temperature range 60–80 °C (Fig. 7). This NMR response to WPI thermal denaturation steadily increased with sodium caseinate concentration. Heating a 3% (w/v) WPI solution containing 1·44% (w/v) κ-casein for 30 min at 90 °C led to an increase in T_2 relaxation rate of 1·5 s^{-1}, i.e. a smaller NMR response than that recorded in the presence of 12% (w/v) sodium caseinate (1·7 s^{-1}).

Replacing sodium caseinate by casein micelles led to the recording of similar but higher NMR responses: no modification of T_2 relaxation rate in 12% casein micelle aqueous solution (14 s^{-1}) was recorded during heating to 90 °C and a rise in this NMR value was observed while heating mixtures of 3% WPI with either 6 or 12% casein micelles between 60 and 80 °C (Fig. 8).

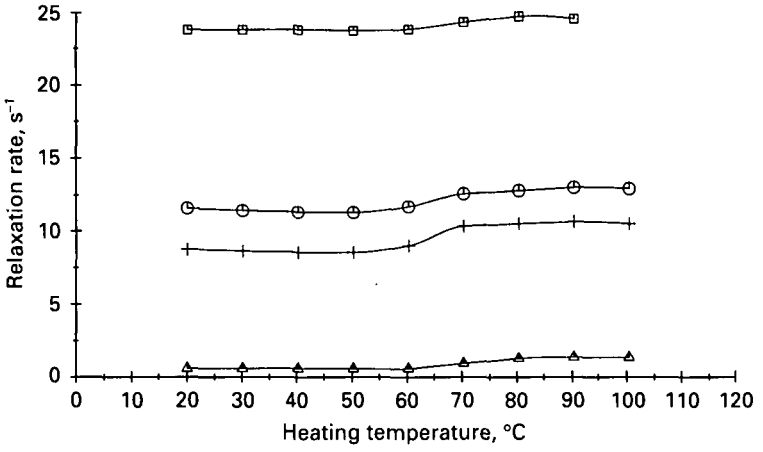

Fig. 8. Water proton T_2 relaxation rates in milk models heated for 30 min successively at increasing temperatures between 20 and 100 °C (10 °C interval). △, 3% whey protein isolate (WPI); +, 3% WPI + 6% casein micelles; ○, 3% WPI + 12% casein micelles; □, dialysed skim milk with 26% dry matter.

Dialysed skim milk

Heating dialysed skim milk (26% dry matter) led to constant T_2 relaxation rates up to 60 °C, increasing rates to 80 °C and then an almost constant rate up to 90 °C (Fig. 8).

DISCUSSION

NMR data measured in dilute whey protein–water systems can be interpreted in terms of a fast exchange between bound and free water populations (Derbyshire, 1982; Padua *et al.* 1991). The finding that the water T_2 proton relaxation was purely exponential in all whey protein–water systems investigated in the present study is consistent with such a fast exchange. It is worth noting that protein–water systems are also characterized by a chemical exchange between labile protein protons and water protons. Such chemical exchange has been postulated in whey protein–water systems (Oakes, 1976*a*; Lambelet *et al.* 1988; Hills *et al.* 1990).

The water proton relaxation rates in aqueous solutions of whey proteins were found to be linearly related to the protein concentrations in the range 0–15% (Lambelet *et al.* 1988, 1989). This indicates that the protein activity is small in this concentration range, and does not contribute to the proton relaxation rate (Kumosinski & Pessen, 1982; Myers-Betts & Baianu, 1990).

It was shown that the protein proton transverse relaxation rate becomes much higher during the thermal denaturation of bovine serum albumin (Oakes, 1976*a*). This indicates that the protein chain becomes much more rigid upon denaturation. The increase in the T_2 relaxation rate of water protons during thermal denaturation of bovine serum albumin was thus explained in terms of enhancement in the bound water proton transverse relaxation rate associated with a decrease in protein chain mobility (Oakes, 1976*a*). The NMR response observed during heating whey protein solutions (Fig. 1) can be understood in a similar way, i.e. can be ascribed to protein unfolding. As no gel was formed under the present conditions the NMR response was not related to gelification.

This NMR response can therefore be used for determining the denaturation following thermal treatment of whey proteins in aqueous solution. A good correlation

was indeed obtained between the NMR and the standard Rowland (1938) methods for measuring whey protein denaturation after thermal treatment (Lambelet *et al.* 1989). The present results (Fig. 3) show that the NMR method is also appropriate for determining whey protein denaturation during isothermal heating.

The influence of salt (KCl or $CaCl_2$), added prior to heating, on the NMR response recorded during whey protein denaturation (Fig. 6) was twofold. First, an increase occurred in the slope of the NMR signal recorded at the beginning of the denaturation process; this slope depended on the nature and on the concentration of the salt, the largest slope being obtained with 33 mM-$CaCl_2$. These effects of KCl and $CaCl_2$ salts on whey thermal denaturation were consistent with those measured with the Rowland (1938) method (Fig. 5). Second, there was an increase in the plateau reached after complete denaturation. Its level was proportional to the ionic strength of the solution as shown by the similarity of the results obtained after 10 h heating in the presence of 33 mM-$CaCl_2$ and 100 mM-KCl. In this case the NMR response cannot be related to denaturation as measured by the Rowland (1938) method. Indeed, with the Rowland (1938) method a degree of denaturation of $\sim 100\%$ is always recorded after 10 h heating. Aggregation of whey proteins during heat treatment is known to be affected primarily by their ionic environment (De Rham & Chanton, 1984; Donovan & Mulvihill, 1987). Although not influenced by gelification, nor by polymerization through the establishment of intermolecular disulphide bonds (Lambelet *et al.* 1989), the T_2 probe is probably sensitive not only to protein unfolding but also to some protein association. This might arise from the addition of salts inducing a decrease in the repulsive forces between proteins: the protein–protein association is improved (Mulvihill *et al.* 1990; Mulvihill & Kinsella, 1988), making the water bound to the protein less mobile.

Casein also influences the NMR probe. The T_2 water proton relaxation in solutions containing 3% whey proteins and 12% sodium caseinate was slow (slightly more rapid than in 3% whey proteins solution) while it was much faster in the presence of 3% whey proteins and 12% casein micelles. This observation, previously reported (Kumosinski *et al.* 1987; Farrell *et al.* 1989), can be explained by the difference in structure between casein micelles (spherical particles with high amounts of interstitial water) and caseinate. At the same protein concentration (3% whey protein and 12% casein micelles) T_2 relaxation was even more rapid in dialysed skim milk, probably owing to the other constituents present in the milk. Although the NMR signal related to protein denaturation in dialysed skim milk was similar to that recorded with the corresponding amount of whey proteins, this signal was increased in the presence of sodium caseinate (Fig. 7) or casein micelles (Fig. 8). This enhancement might be associated with the formation of a complex on heating β-lactoglobulin with κ-casein (Reddy & Kinsella, 1990). However, this complex cannot account for the entire increase observed. Indeed, κ-casein added to whey proteins in an amount equal to that present in sodium caseinate produced a smaller effect than did sodium caseinate.

It appears that, like thermal denaturation or aggregation, the NMR response is influenced by environmental factors (ionic strength, casein). The NMR method cannot therefore be universally applied. Like the standard Rowland (1938) solubility assay (De Rham & Chanton, 1984), the NMR method is valid under restricted conditions. Nevertheless, it is a powerful tool for studying thermal denaturation of pure whey proteins, whey protein concentrates or isolates. Using the NMR method, denaturation can be measured either discontinuously by recording values at 20 °C after the thermal treatments or by continuously sampling information above the

denaturing onset temperature. As a matter of fact, under these conditions the effect of temperature on the relaxation rates is cancelled. Furthermore, as the relaxation curves can be recorded semi-automatically during isothermal treatment, the NMR method is very convenient for following denaturation at a constant temperature.

No attempt was made to calculate kinetic information from the whey proteins denaturation curves obtained with the NMR method (Fig. 3) since the relative denaturation rates of the individual proteins differ (Donovan & Mulvihill, 1987). The NMR method is, however, appropriate for kinetic determinations. On the other hand, while irreversible denaturation is observed when the measures are made at 20 °C, reversible effects can be detected when the measures are made above the denaturing onset temperature. This could be useful for studying heat-induced denaturation of e.g. β-lactoglobulin and α-lactalbumin.

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REFERENCES

Association of Official Analytical Chemists 1980 *Official Methods of Analysis of the AOAC*, 13th edn, Methods 16.047, 16.048 and 47.021 (Ed. W. Horwitz). Washington, DC: AOAC

Blicharska, B. & Rydzy, M. 1979 Investigation of protein denaturation by nuclear magnetic relaxation method. *Acta Physica Polonica* **A56** 439–443

Derbyshire, W. 1982 The dynamics of water in heterogeneous systems with emphasis on subzero temperatures. In *Water: a Comprehensive Treatise*, vol. 7, pp 339–430 (Ed. F. Franks). New York: Plenum Press

De Rham, O. & Chanton, S. 1984 Role of ionic environment in insolubilization of whey protein during heat treatment of whey products. *Journal of Dairy Science* **67** 939–949

De Wit, J. N. 1981 Structure and functional behaviour of whey proteins. *Netherlands Milk and Dairy Journal* **35** 47–64

Donovan, M. & Mulvihill, D. M. 1987 Thermal denaturation and aggregation of whey proteins. *Irish Journal of Food Science and Technology* **11** 87–100

Farrell, H. M., Pessen, H. & Kumosinski, T. F. 1989 Water interactions with bovine caseins by hydrogen-2 nuclear magnetic resonance relaxation studies: structural implications. *Journal of Dairy Science* **72** 562–574

Goldsmith, S. M. & Toledo, R. T. 1985 Studies on egg albumen gelation using nuclear magnetic resonance. *Journal of Food Science* **50** 59–62

Hills, B. P., Takacs, S. F. & Belton P. S. 1990 A new interpretation of proton NMR relaxation time measurements of water in food. *Food Chemistry* **37** 95–111

Kumosinski, T. F. & Pessen, H. 1982 A deuteron and proton magnetic resonance relaxation study of β-lactoglobulin A association: some approaches to the Scatchard hydration of globular proteins. *Archives of Biochemistry and Biophysics* **218** 286–302

Kumosinski, T. F., Pessen, H., Prestrelski, S. J. & Farrell, H. M. 1987 Water interactions with varying molecular states of bovine casein: ^{2}H NMR relaxation studies. *Archives of Biochemistry and Biophysics* **257** 259–268

Lambelet, P., Berrocal, R., Desarzens, C., Froehlicher, I. & Ducret, F. 1988 Pulsed low-resolution NMR investigations of protein sols and gels. *Journal of Food Science* **53** 943–946, 964

Lambelet, P., Berrocal, R. & Ducret, F. 1989 Low resolution NMR spectroscopy: a tool to study protein denaturation. I. Application to diamagnetic whey proteins. *Journal of Dairy Research* **56** 211–222

Lambelet, P., Ducret, F., Leuba, J. L. & Geoffroy, M. 1991 Low-field NMR relaxation study of the thermal denaturation of transferrins. *Journal of Agricultural and Food Chemistry* **39** 287–292

Mulvihill, D. M. & Donovan, M. 1987 Whey proteins and their thermal denaturation – a review. *Irish Journal of Food Science and Technology* **11** 43–75

Mulvihill, D. M. & Kinsella, J. E. 1988 Gelation of β-lactoglobulin: effects of sodium chloride and calcium chloride on the rheological and structural properties of gels. *Journal of Food Science* **53** 231–236

Mulvihill, D. M., Rector, D. & Kinsella, J. E. 1990 Effects of structuring and destructuring anionic ions on the rheological properties of thermally-induced β-lactoglobulin gels. *Food Hydrocolloids* **4** 267–276

Myers-Betts, P. A. & Baianu, I. C. 1990 Approaches to protein hydration and water activity in food proteins by nuclear magnetic relaxation and vapor pressure equilibration techniques. *Journal of Agricultural and Food Chemistry* **38** 1171–1177

Oakes, J. 1976*a* Protein hydration. Nuclear magnetic resonance relaxation studies of the state of water in native bovine serum albumin solutions. *Journal of the Chemical Society, Faraday Transactions I* **72** 216–227

Oakes, J. 1976*b* Thermally denatured proteins. Nuclear magnetic resonance, binding isotherm and chemical modification studies of thermally denatured bovine serum albumin. *Journal of the Chemical Society, Faraday Transactions I* **72** 228–237

PADUA, G. W., RICHARDSON, S. J. & STEINBERG, M. P. 1991 Water associated with whey protein investigated by pulsed NMR. *Journal of Food Science* **56** 1557–1561

PUMPERNIK, D., FAJT, B., LAPANJE, S. & AŽMAN, A. 1975 NMR relaxation study of the thermal denaturation of lysozyme. *Zeitschrift für Naturforschung* **30C** 294–295

REDDY, I. M. & KINSELLA, J. E. 1990 Interaction of β-lactoglobulin with κ-casein in micelles as assessed by chymosin hydrolysis: effect of temperature, heating time, β-lactoglobulin concentration, and pH. *Journal of Agricultural and Food Chemistry* **38** 50–58

ROWLAND, S. J. 1938 The determination of the nitrogen distribution in milk. *Journal of Dairy Research* **9** 42–46

RYDZY, M. & SKRZYNSKI, W. 1980 Investigation of β-galactosidase thermal denaturation by nuclear magnetic relaxation method. *Studia Biophysica* **78** 119–126